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ALGAL DEGRADATION IN WATERWAYS UNDER ANAEROBIC CONDITIONS

Perry L. McCarty, et al

Stanford University

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Progress Report

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June 1, 1967 through May 31, 1968

U.S. Department of the Interior
Federal Water Pollution Control Administration
Research Grant WP 01037

Principal Investigator

Perry L. McCarty
Professor of Sanitary Engineering

Co-investigator

Edward G. Foree
Research Assistant

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Stanford, California 94305

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ALGAE DEGRADATION IN WATERWAYS
ANAEROBIC CONDITIONS

The investigation financed by Research Grant WP-01037 from the Federal Water Pollution Control Administration is being conducted in two distinct phases. One phase is a study of the degradation of algae under aerobic conditions, and the second is a similar study, but under anaerobic conditions. The aerobic study has essentially been completed and is covered in detail in a separate report. The anaerobic study, which is the subject of this report, is still underway and will be completed during the coming third year of project support. The following is a very brief summary of the important aspects of the anaerobic study which have been completed to date.

I. INTRODUCTION

The discharge of inorganic nutrients into waterways is producing a variety of problems, perhaps the most significant being the acceleration of the rate of eutrophication. One of the most significant problems associated with eutrophication is excessive growth of algae. The nutrients nitrogen and phosphorus have been found to be the most serious offenders in promoting such growth. One or both are usually found to limit algal growth in natural bodies of water, as most other required nutrients are normally present in sufficient quantities.

Algae are the major primary producers of organic matter in natural waters. The presence of organic matter, and more importantly, its resulting decay, produce many problems associated with water quality and its control. Algae, being autotrophic-photosynthetic organisms, do not require organic matter for a sources of energy and for cell synthesis, but utilize sunlight as a source of energy, and inorganic carbon compounds and other inorganic nutrients for cell synthesis. Every gram of cellular organic matter thus produced must ultimately be decomposed. This decomposition, however, may vary in rate and manner, depending primarily upon the environmental conditions present.

There are two general characteristics of an aquatic environment in which algal growth and/or decay might occur: (1) that characterized by the presence or absence of dissolved oxygen, the environment being respectively termed aerobic or anaerobic, and (2) that characterized by the presence or absence of light, the environment being respectively termed light or dark. Based on these two parameters, four possible sets of conditions arise: light-aerobic, light-anaerobic, dark-aerobic, and dark-anaerobic.

The light-aerobic environment represents a condition favorable for photosynthesis. Respiration also occurs here, but under most natural circumstances the rate of photosynthesis is much greater than the rate of respiration. The resulting net production of oxygen represents a contribution to the oxygen resources of a body of water, and from this standpoint, may be desirable.

The light-anaerobic condition is rare in natural waters since in the presence of light algae will carry on photosynthesis, hence producing oxygen and creating an aerobic environment. This condition might occur early in the morning in a body of water characterized by a dense growth of algae which, through respiration, had depleted the dissolved oxygen supply during the night.

The dark-aerobic environment is characterized by algal respiration and decay. This decay causes a very real oxygen demand which is detrimental to water quality. The characteristics of decay under these conditions was studied under this research grant and the results are summarized in an accompanying report.

The dark-anaerobic condition describes an environment in which in general only algal death and decomposition can occur. A review of the literature showed that very little research yielding quantitative results describing the processes which occur in such an environment has been conducted. It is believed that the biological, chemical, and physical changes which take place can have significant effects on water quality. One of the purposes of this research was to study in detail the phenomena which occur in such an environment, to determine the significance of the various environmental factors

which affect these phenomena, and to evaluate the resulting effects on water quality.

A dark-anaerobic environment occurs in several locations in surface waters. It occurs in the bottom deposits of the majority of natural lakes and man-made reservoirs, especially in highly productive lakes where large quantities of organic matter settle to the bottom. Large portions of the hypolimnion in stratified eutrophic lakes and reservoirs are anaerobic during extended periods of summer stratification. Sewage and industrial waste treatment lagoons, especially those termed as "facultative," are characterized by an anaerobic lower layer into which much of the algae and other biological and organic solids settle. This condition can also exist in large sluggish streams and estuaries, although, because of turbulance and the resulting mixing, the condition does not occur as frequently here as in the more quiescent lakes and reservoirs.

Algae are being considered as a possible means of removal of nutrients from nutrient-rich waters. For such schemes to fulfill their purpose the algal mass must be harvested from the water being treated and be disposed of in some economical manner. Anaerobic digestion has been proposed by some as a possible method of treatment prior to ultimate disposal. Although there have been some studies on the anaerobic digestion of algae, detailed quantitative information describing the processes involved is lacking. It is believed that the results of this research will contribute to a more thorough understanding of these processes.

The major objectives of the research covered by this progress report were to determine the rates and extent of algal degradation under dark-anaerobic conditions, and to evaluate the effects of important biological and environmental factors on this decomposition. The principal processes studied were the conversion of particulate algal organics to soluble organics, the stabilization of the organic matter as measured by reduction in its chemical oxygen demand, and the regeneration of nitrogen and phosphorus during decomposition. The primary biological and environmental factors studied were bacterial seeding, algal species and cell composition, inorganic medium composition, temperature, and pH. A summary of the progress of this particular study is contained in the following.

II. THEORETICAL CONSIDERATIONS

In natural situations, algae enter dark-anaerobic environments primarily by gravitational sedimentation, where, due to environmental conditions unfavorable for their growth, they die and decompose. Two general aspects of this algal decomposition are significant in relation to water quality: (1) the decomposition of the algal organic matter as measured by changes in its oxygen demanding capacity, and (2) the associated regeneration (release to solution) of important algal growth nutrients, primarily nitrogen and phosphorus.

A. Anaerobic Decomposition of Algal Organics

In general, under dark-anaerobic conditions, algae cannot carry on any significant degree of metabolic activity and, after a relatively short period of exposure, die. Algal cells undergo autolysis and are also subject to attack by anaerobic bacteria which utilize the algal organics as sources of energy and material for cell synthesis.

The primary microbial process responsible for the stabilization of organic matter under anaerobic conditions is methane fermentation. The overall methane fermentation process may be described as occurring in two stages as shown below.

$$\text{Complex Organics} \xrightarrow[\text{Acid Formation}]{\text{Bacteria}} \text{Organic Acids} \xrightarrow[\text{Methane Formation}]{\text{Bacteria}} CH_4 + CO_2$$

During the first stage, a heterogeneous group of facultative and anaerobic bacteria converts complex organics to organic acids by hydrolysis and fermentation. Complex proteins are first hydrolyzed with the aid of enzymes to polypeptides and then to simple amino acids; complex

carbohydrates such as starch and cellulose are hydrolyzed to simple sugars; and complex lipids are hydrolyzed to glycerol and fatty acids. These hydrolysis products are then fermented by the acid-forming bacteria. This fermentation consists of the oxidation and reduction of organic matter saturated fatty acids and carbon dioxide representing the reduced and oxidized products, respectively. Ammonia is also an end product of amino acid fermentation.

During the second stage, a group of anaerobic bacteria utilize the organic acids, converting them to methane and carbon dioxide.

The first stage conversion by the acid-forming bacteria occurs with little or no change in the chemical oxygen demand (COD) of the organic matter; simply a change in form takes place, a portion being converted to end products such as organic acids and the other protion being converted to bacterial cells. It is only in the second stage (methane fermentation) that removal or stabilization of oxidizable organics occurs and the quantity removed is directly proportional to the quantity of methane produced. The oxygen equivalent of organic matter removed by methane fermentation can be determined from the following relationship:

$$CH_4 + 2O_2 \longrightarrow 2CO_2 + 2H_2O$$

M.W. 16 64

Each gram of methane produced and lost to the atmosphere corresponds to 4 grams of COD equivalent of organic matter removed. Appropriate calculations yield the following equivalence: 1 ml of methane produced at STP corresponds to 2.86 mg of COD removal.

In the presence of potential inorganic electron acceptors other than carbon dioxide, other microbial processes can be responsible for the decomposition of significant quantities of organic matter under anaerobic conditions. The most significant of these potential electron acceptors in natural environments is sulfate, which can be used by certain anaerobic heterotrophic bacteria in the metabolic oxidation or organic matter in the absence of free oxygen. The overall reaction can be described in the most

general terms by the following expression:

$$SO_4^{=} + \text{Organic Matter} \xrightarrow{\text{Bacteria}} S^{=} + H_2O + CO_2$$

Appropriate calculations yield the following equivalence: 1.5 mg of sulfate (as SO_4) reduced to sulfide is equivalent to the oxidation of 1.0 mg of organic COD.

B. Nutrient Regeneration

The net release of nutrients to solution during the bacterial decomposition of algal material is the resultant of two components: a positive contribution due to the release from the algal material and a negative contribution due to bacterial synthesis. Under anaerobic conditions favorable for bacterial activity, nitrogen is regenerated primarily as ammonia nitrogen and phosphorus is regenerated as inorganic phosphates. Soluble organic nitrogen and soluble organic phosphorus may also be present in varying quantities during decomposition, these quantities being relatively small under normal environmental conditions.

The rate and extent of regeneration of nutrients are primarily dependent upon the rate and extent of decomposition of the algal organics, the nutrient content of the algal material, and any characteristics of the medium which may affect the regeneration process chemically. Examples of the latter are pH and the presence of ions which form insoluble precipitates with the nutrient released from the algal material.

C. Chemical Composition of Algae

A consideration which is very important to both the decomposition of algal organic matter and the regeneration of nutrients is the chemical composition of algae. Accordingly, a review of the literature was conducted to find previously reported values for the chemical composition of various freshwater algae.

The most significant finding was that the chemical composition of algae, especially freshwater green and blue-green algae, is much more dependent upon environmental conditions than upon taxonomic position. In order to illustrate this, a summary of the results of a study by Spoehr and Milner (1) is presented in Table 1. It was assumed that the major elemental constituents (C, H, O, and N) comprised 100 percent of the ash-free dry weight and that the sum of the protein, carbohydrate, and lipid fractions was equal to 100 percent of the ash-free dry weight. The ash content shown was expressed as a percentage of the total dry weight. The purpose of their study was to determine the effect of environmental conditions on the chemical composition of Chlorella pyrenoidosa. The extreme variation, especially with respect to the protein, carbohydrate, and lipid contents, is obvious. Cells of high lipid content were produced in cultures grown in media with low nitrogen concentrations and in cultures illuminated with high light intensity. It was also found that, over the range studied, the phosphorus concentration did not significantly affect the organic composition of the cells and that cells with high lipid content carried on photosynthesis with a chlorophyll content of 1/500 to 1/2000 that of cells of low lipid content.

Table 2 also indicates the wide range of concentrations of several minor inorganic chemical constituents reported for Chlorella pyrenoidosa. The algae represented were grown under a variety of environmental conditions in both batch and continuous culture.

A knowledge of the chemical composition of algae is important in a study of their anaerobic bacterial decomposition in several respects. Different classes of organic compounds have different anaerobic decomposition schemes. For example, consider the overall methane fermentation process for proteins, carbohydrates, and lipids. The decomposition processes can differ among these groups of organics with respect to time rates, identity of the bacteria involved, identity and quantities of volatile acid intermediates produced, amount of bacterial synthesis, and relative quantities of stable end products produced. The quantities of the nutrients, nitrogen

TABLE 1

CHEMICAL COMPOSITION OF *CHLORELLA PYRENOIDOSA* GROWN UNDER VARIOUS ENVIRONMENTAL CONDITIONS. [Based on data from Spoehr and Milner (1)] .

	(% Dry Wt.)	(% Ash-Free Dry Wt.)						
Culture	Ash	C	H	O	N	Protein	Carbohydrate	Lipid
1	3.45	49.5	6.78	34.4	9.31	58.0	37.5	4.5
2	3.78	51.0	6.90	30.8	11.29	70.5	23.8	5.7
3	20.21	51.6	7.37	26.9	14.11	88.2	6.6	5.2
4	5.81	54.6	7.28	27.7	10.38	64.8	17.7	17.5
5	8.56	54.8	7.93	32.0	5.28	33.0	38.2	28.8
6	7.88	54.6	8.11	26.6	10.75	67.2	11.4	21.4
7	2.28	56.0	7.84	28.3	7.86	49.2	23.0	27.8
8	2.87	59.7	8.56	26.9	4.80	30.0	24.8	45.2
9	1.36	63.8	8.97	24.2	3.08	19.2	20.8	60.0
10	5.32	65.4	9.68	23.3	1.62	10.1	20.0	69.9
11	4.57	68.2	10.01	20.5	1.28	8.0	13.2	78.8
12	3.44	69.2	10.33	19.3	1.17	7.3	9.5	83.2
13	3.46	70.2	10.53	17.9	1.43	8.9	4.8	86.3
Mean	5.60	59.1	8.49	26.1	6.33	39.6	19.3	41.1

TABLE 2

RANGES OF CONCENTRATIONS OF MINOR CHEMICAL CONSTITUENTS IN *CHLORELLA PYRENOIDOSA* CELLS

Constituent	(% of Total Dry Weight) Concentration Range	Reference
Total Ash	5.12-8.66	Scott (2)
P	0.94-1.51	Scott (2)
Ca	0.00-1.55	Scott (2)
Mg	0.26-1.50	Scott (2)
K	0.04-1.43	Scott (2)
Na	0.07-0.70	Scott (2)
P	1.35-2.76	Knauss and Porter (3)
S	0.42-0.77	Knauss and Porter (3)
Ca	0.01-0.40	Knauss and Porter (3)
Fe	0.02-3.45	Knauss and Porter (3)
Mn	0.02-2.60	Knauss and Porter (3)
Sr	0.0004-0.051	Knauss and Porter (3)
Cu	0.0008-0.034	Knauss and Porter (3)
Zn	0.0004-0.0009	Knauss and Porter (3)
Total Ash	10.50-14.32	Ketchum and Redfield (4)
P	2.43-3.01	Ketchum and Redfield (4)
Total Ash	1.36-20.21	Spoehr and Milner (1)

and phosphorus, regenerated during decomposition is obviously directly related to the algal cell content of these nutrients.

III. EXPERIMENTAL PROCEDURES

The principal portion of this research consists of laboratory studies of algal cultures decomposing under dark, anaerobic, constant temperature conditions. The decomposing cultures were analyzed periodically for COD, suspended solids, pH, various forms of nitrogen and phosphorus, total volatile acids, and other parameters in some experiments. In general, the analytical techniques utilized in these analyses were those commonly employed in water quality control work and for the most part conform to the procedures described in Standard Methods for the Examination of Water and Wastewater, Twelfth Edition (5). In some of the experiments individual volatile acids were identified and quantatively measured utilizing a gas chromatographic procedure, and gases produced were identified and quantitatively measured utilizing a gas chromatographic procedure combined with a manometric procedure. The chromatographic procedures followed were those previously described by O'Rourke (6).

The algae for the majority of the studies were grown in the laboratory in a constant temperature room at 20° C, provided with artificial illumination by daylight fluorescent lights, and provided with a gas mixture of 2 percent carbon dioxide and 98 percent air which was continually bubbled through the growing cultures. The algae were grown as pure (bacteria-free), unialgal, and heterogeneous cultures. The algae used in the pure and unialgal studies were obtained from "The Culture Collection of Algae at Indiana University" (7). The algae for the heterogeneous cultures were originally obtained from ponds in Kentucky.

The majority of the algae were grown in synthetic media as presented in Table 3. In one study algae were grown in several natural waters to which nitrogen and phosphorus were added in the concentrations given

for the above medium. It was found that more favorable pH conditions could be maintained during the anaerobic decomposition studies if the bicarbonate alkalinity of the above medium were increased to 500 mg/l at the initiation of anaerobic conditions. The algal cultures were allowed to decompose in the same media in which they were grown with the exception of the addition of the bicarbonate alkalinity mentioned above and the addition of biological seeding materials. Small quantities of lake bottom deposits, digested municipal waste sludge, settled municipal wastewater, and samples from previously decomposing units were sometimes added at the initiation of anaerobic conditions to provide bacteria seed.

Algae for one series of experiments were collected from a pond at Los Gatos, California, and then resuspended in a synthetic medium.

All cultures were placed for decomposition in 9-liter vessels as shown schematically in Figure 1. Most of the cultures were grown in these same vessels, the exceptions being a few which were originally grown in 2.8 liter culture vessels and then transferred to the 9-liter units for the study of decomposition. Port A was utilized for the addition of samples, media, and gases; Port B, for withdrawing gas; and Port C, for withdrawing liquid samples while the contents were being stirred by a magnetic stirrer.

At the beginning of the anaerobic decomposition studies, the cultures were rendered anaerobic by purging with nitrogen gas for several minutes; the seeding material, if any, was added; and the vessels were sealed and placed in dark cabinets in a constant temperature room for incubation.

TABLE 3

CHEMICAL COMPOSITION OF SYNTHETIC ALGAL GROWTH MEDIUM

Chemical	Concentration (mg/l)	Comments
$MgSO_4 \cdot 7H_2O$	45.0	
$CaCl_2$	55.0	
$NaHCO_3$	250	200 mg/l Alkalinity as $CaCO_3$
$KHCO_3$	100	
KH_2PO_4	28.1	5.00 mg/l P
NH_4Cl	95.5	25.0 mg/l N
Fe, B, Mn, Mo	0.1	Added as 1 ml of a trace element solution per liter of medium
Zn, Cu, Co, Ni, Cr, V	0.01	
EDTA	2.0	
KOH	1.0	

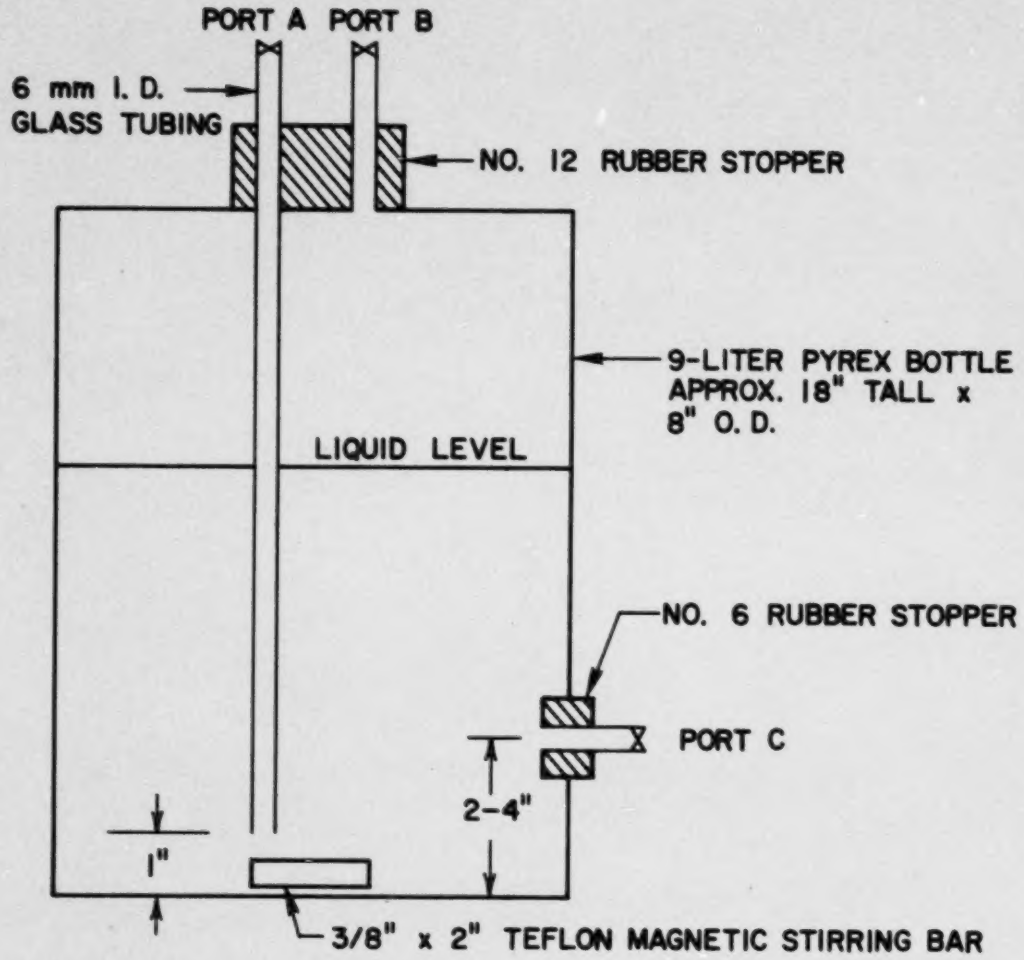

FIGURE 1. Schematic Diagram of Vessel Used for Anaerobic Decomposition Studies.

IV. RESULTS

A. General Comments

The results presented in this section are based on data resulting from analyses of samples periodically removed fr.m the anaerobically decomposing algal cultures.

In the subsequent presentation numbers are used for reference to the various cultures. These numbers were used for identification purposes during the experiments. The numbers in paraentheses following the names of the unialgal cultures are those used for identification purposes in the Indiana University Collection.

For the sake of brevity, certain abbreviations will be used as defined in Table 4. Also included are the means by which each of the values represented were determined.

TABLE 4

SUMMARY OF ABBREVIATED SYMBOLS USED

Abbreviation	Definition	Means of Determination
TCOD	Total Chemical Oxygen Demand	Direct Measurement
SCOD	Soluble Chemical Oxygen Demand	Direct Measurement
PCOD	Particulate Chemical Oxygen Demand	Calculated as TCOD-SCOD
VSS	Volatile Suspended Solids	Direct Measurement
TKN	Total Kjeldahl Nitrogen	Direct Measurement
SKN	Soluble Kjeldahl Nitrogen	Direct Measurement
PN	Particulate Nitrogen	Calculated as TKN-SKN

The time scale which will be indicated is that since the imposition of anaerobic conditions in the cultures.

The final results of the experimental studies are to be divided into three major divisions: those related to the decomposition of algal organic matter, those related to the regeneration of nitrogen and phosphorus, and those from miscellaneous studies. The study related to the decomposition of organic matter was divided into two parts: a study of decomposition in which methane fermentation was the mechanism responsible for the stabilization of COD, and a study of decomposition in which sulfate reduction was the mechanism responsible. Presented in this progress report is only a portion of the results related to the decomposition of algal organic matter in which methane fermentation was the major mechanism. The nutrient regeneration studies and evaluation and the sulfate reduction phase are presently in progress and will be completed during the coming terminal year of this project.

B. Decomposition of Algal Organics by Methane Fermentation

As previously indicated the overall methane fermentation processes is comprised of two principal stages: a conversion of complex organics to volatile organic acids and the conversion of these acids to methane and carbon dioxide.

Golueke *et al.* (8) have demonstrated that algal organics (primarily *Chlorella* and *Scenedesmus* cells) can be stablized to some degree by methane fermentation under the conditions normally employed for the anaerobic digestion of domestic waste sludge, *viz.*, high temperature, high organic concentration, and large population of acclimated bacteria. However, quantitative information on the methane fermentation of algal organics under conditions more closely simulating those which exist in natural environments is lacking. One of the objectives of this research was to determine the significance of methane fermentation for the stabilization of algal organics under such conditions.

Methane fermentation was found to be the mechanism responsible for the stabilization of algal organics in all of the cultures studied with the following exceptions: (1) those with pH values low enough to be toxic to the methane bacteria, (2) the pure algal cultures from which all bacteria were excluded, and (3) those with high concentrations of sulfate. No significant stabilization was observed in (1) and (2) and sulfate-reduction was the mechanism responsible for the stabilization in (3).

The results also demonstrated that a significant portion of algal organics can be stabilized by methane fermentation, a stabilization as high as 80 percent of the total initial COD having been accomplished in several of the cultures.

The purpose of the results presented in this section are to demonstrate the relationships between methane production and reduction of total COD in a decomposing algal culture and to indicate the relative quantities of the various volatile acids present during the decomposition. Results from Culture 9 are used for illustration. This was a culture of algae collected from a pond and seeded at the beginning of decomposition with a small quantity (1.7 ml seed per liter of culture) of material from a previously decomposing culture (Culture 4). Although the rates and degrees of conversion of algal organics to soluble organics (as measured by decreases in PCOD) and stabilization of organic matter (as measured by decreases in TCOD) varied from culture to culture depending on various factors to be discussed later, the relationships between methane production and COD reduction and the relative quantities of the various volatile acids produced in Culture 9 are typical of all of the cultures in which methane fermentation occurred.

Presented in Figure 2 are the cumulative volumetric productions of methane and carbon dioxide over a period of decomposition of 341 days. Also shown is the measured reduction in the TCOD concentration in the culture over the same period. The values for TCOD reduction were calculated by subtracting the measured TCOD at a given time from the initial TCOD (3200 mg/1).

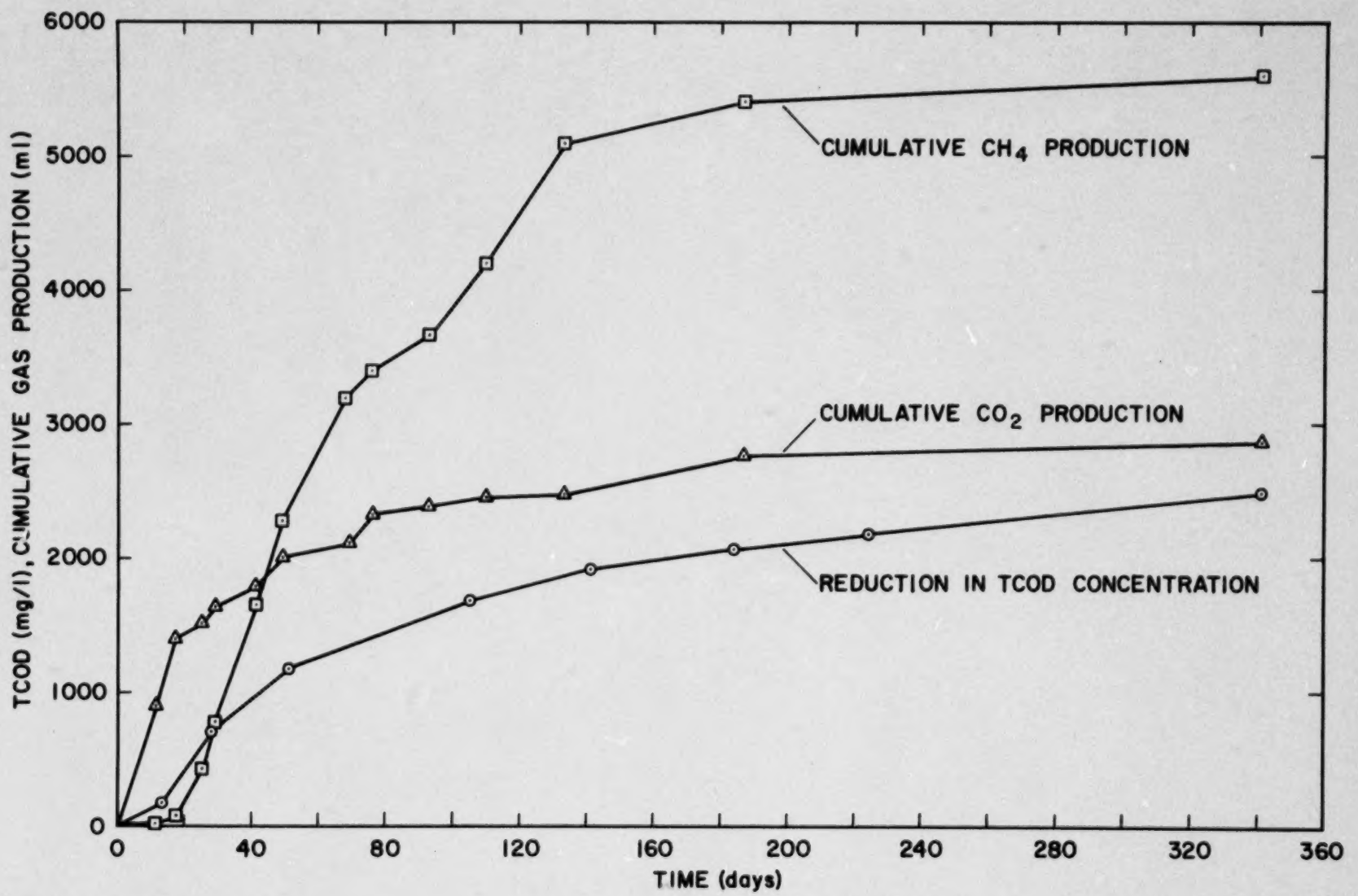

FIGURE 2. Gas Production and TCOD Reduction in Culture No. 9.

A precise calculation of the equivalence between the theoretical COD of the methane produced and the measured reduction in TCOD in the culture over the entire period of decomposition could not be made because the volume of the liquid in the vessel was changing with time due to the removal of samples for analyses. However, the quantity of liquid volume change was small in comparison to the total liquid volume, and it was considered to be a good assumption to take an effective liquid volume as being that at the time when the cumulative methane production was 1/2 of the total. This liquid volume was 5720 ml at 60 days compared to 6000 ml at the beginning of decomposition. Using this value and the measured decrease in TCOD concentration of 2490 mg/l, the total measured COD reduction over the 341 day period was 14,200 mg. Using the measured total volume of methane produced of 5600 ml and the theoretical factor of 2.66 mg COD per ml methane at 1 atm pressure and 20° C (the experimental conditions of gas measurement), the calculated COD equivalent of the methane produced over the 341 day period is 14,900 mg. The percent difference between these two values is 4.8 percent, which is not considered to be greater than that introduced as a result of the above assumption and errors in measuring gases, etc. Similar equivalences were demonstrated for all of the cultures for which cumulative methane productions were measured.

A plot of the concentrations of individual acids (expressed on a theoretical COD basis) present in Culture 9 during decomposition is presented in Figure 3. Also shown are the total volatile acid COD (calculated as the sum of the individual volatile acid COD values) and the measured soluble COD (SCOD) concentrations. The difference between SCOD and total volatile acid COD is an indication of the presence of soluble organics other than volatile acids. The quantity of other soluble organics in most cultures was small.

The volatile acids concentrations present represent the net result of production by the acid-forming bacteria and utilization by the methane-forming bacteria. Except for the two exceptions mentioned below, the pattern of variation of the individual volatile acids with time is typical

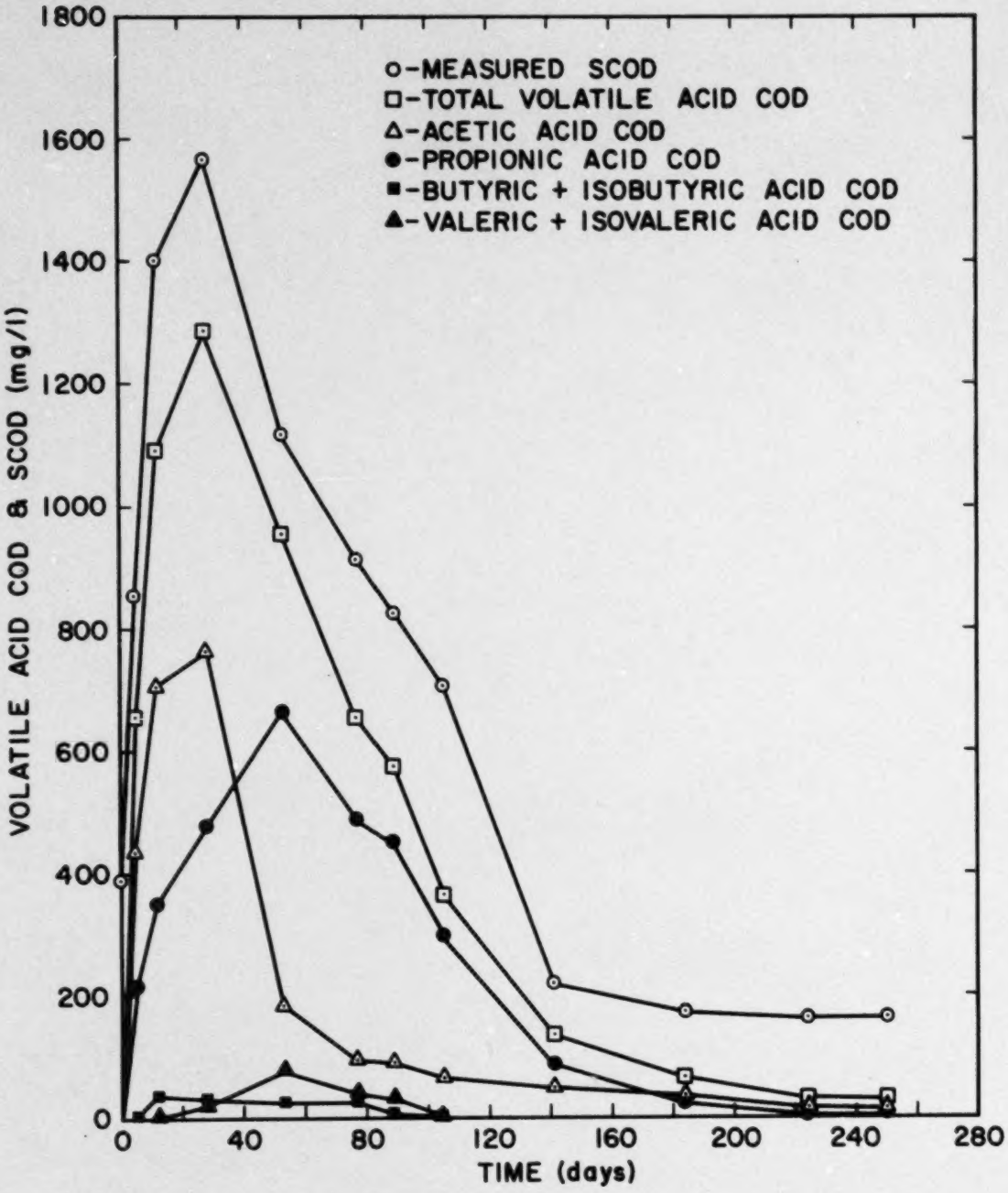

FIGURE 3. Individual and Total Volatile Acids COD and Measured SCOD Concentrations Present During Decomposition in Culture 1.

of all of the cultures in which methane fermentation occurred. The typical pattern of variation consisted of an initial relatively rapid build-up of acetic acid with a lesser build-up of propionic acid, followed by a relatively rapid net utilization of acetic acid and a slower net utilization of propionic acid. Measurable quantities of butyric, isobutyric, valeric, and isovaleric acids were also present during decomposition in most of the cultures, but these quantities were small compared to the quantities of acetic and propionic acids present and were relatively readily utilized.

The relative rates of utilization of acetic and propionic acids were different in the cultures incubated at 15° C and 25° C, and acetic acid was the only volatile acid present in the cultures in which sulfate reduction was occurring.

C. Effects of Biological and Environmental Factors

Presented in this section are results of studies to determine the effects of various factors on the decomposition of algal organics. The reduction of PCOD in the cultures was the combined result of release of soluble organics from algal cells during autolysis and the conversion of particulate organics to soluble organics by bacteria. Bacterial synthesis also resulted in a small positive contribution to the PCOD in the cultures. Previous studies have shown that the percentage of substrate COD which is converted into bacterial cells is relatively small under anaerobic conditions. Although it depends on chemical composition of the substrate, it was estimated that this value was about 10 percent or less for the complex algal organics, and that the COD of the bacterial solids present in the decomposing cultures at any time was less than 5 percent of the total initial PCOD. No attempt was made in any of the studies to separate the algal and bacterial solids.

The initial COD concentrations in most of the cultures were on the order of 1000 mg/l. Cultures 1, 2, and 3 were exceptions, having initial COD's of about 10,000 mg/l.

Because the absolute values varied from culture to culture, the TCOD and PCOD values presented in the following sections have been computed on a more comparable percentage basis as follows:

$$\text{TCOD (\% Remaining)}_t = 100\ \text{TCOD}_t/\text{TCOD}_o, \text{ and}$$

$$\text{PCOD (\% Remaining)}_t = 100\ \text{PCOD}_t/\text{PCOD}_o,$$

where the subscripts represent time.

1. Bacterial Seeding. A sample of algae was collected from a pond, suspended in a synthetic medium, and divided into three equal aliquots. No seeding material was added to one aliquot (Culture 1), 17 ml of bottom deposit collected from a eutrophic lake was added per liter to the second aliquot (Culture 2) and 17 ml of sludge taken from an anaerobic digester treating domestic waste sludge was added per liter to the third aliquot (Culture 3).

A plot showing the stabilization of TCOD with decomposition time in these cultures is presented in Figure 4. The methane bacteria which were responsible for the TCOD stabilization characteristically have a long generation time. It was expected that the largest population of these methane bacteria would initially be present in Culture 3 (seeded with the anaerobic sludge), the second largest population in Culture 2 (seeded with the lake deposit), and the smallest population in Culture 1 (no additional seed added). This was confirmed by the longer initial lag in TCOD stabilization in Culture 2 than in Culture 3 and in Culture 1 than in Culture 2. During these time lags the populations of methane bacteria were increasing to sizes sufficient to accomplish a significant degree of methane fermentation. The long-term extent of TCOD stabilization was not significantly different for the three cultures. About 30 percent of the initial TCOD remained as refractory organics after a year of decomposition. These results indicate that all species of bacteria required for the stabilization of the algal organics were present in all of the cultures; only the initial total population levels were different.

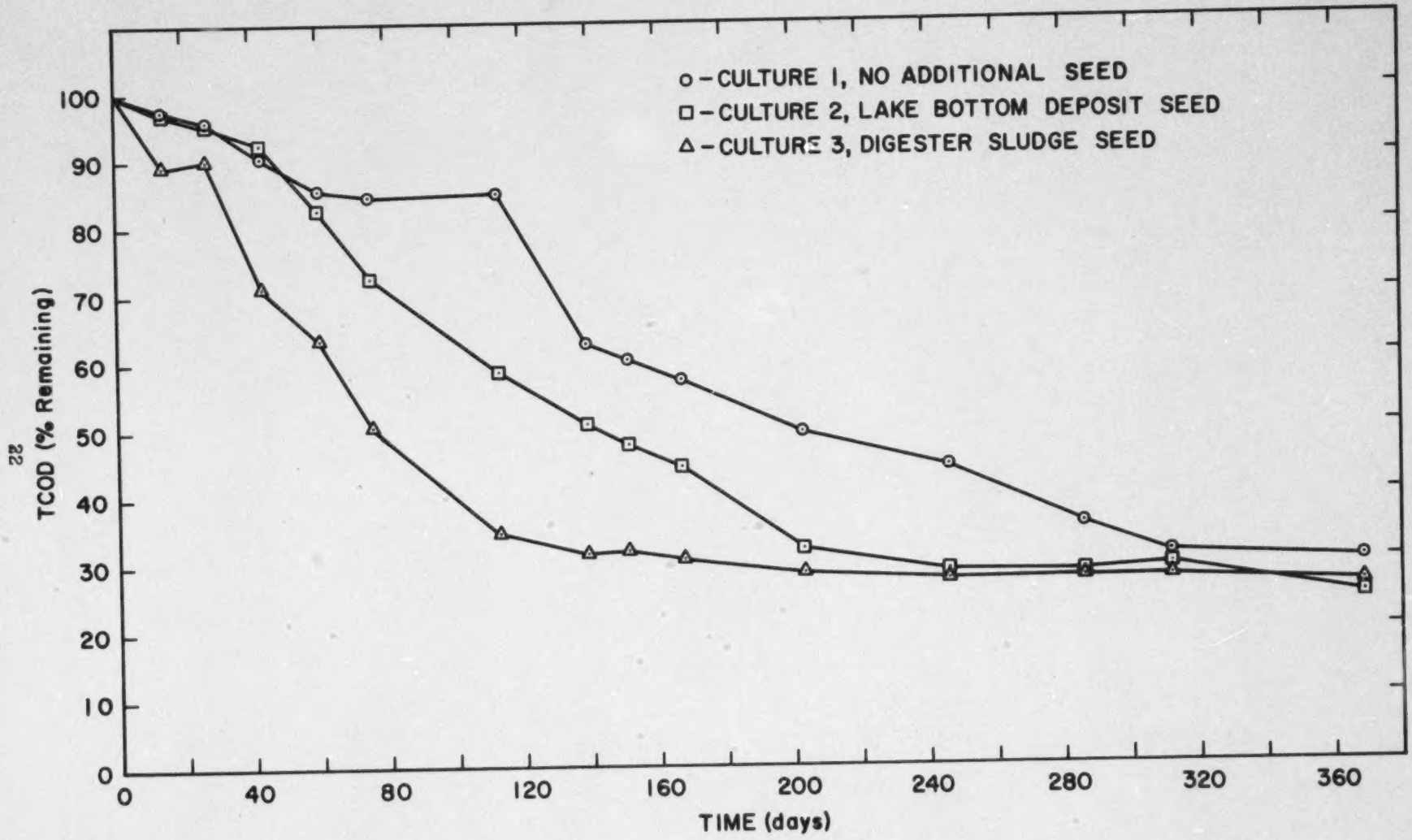

FIGURE 4. Effects of Bacterial Seeding on TCOD Stabilization in Decomposing Cultures of Pond Algae.

A plot showing the reduction of PCOD during decomposition in these cultures is presented in Figure 5. No significant differences either in rates or in extent of long-term reduction in PCOD were noted with variation in seedings in these cultures.

These results brought up the question as to what if any roll the bacteria might play in the initial conversion of particulate algal organics to soluble organics since no significant differences were observed in the reduction of PCOD with the three different bacterial seeds used in this study. A large majority of the soluble organics present were in the form of volatile acids, but it was not known whether these acids were formed from organics released by autolysis of the algal cells or if the bacteria were directly responsible for the conversion of the particulate algal organics to soluble organics.

In order to investigate this question a culture of Chlorella pyrenoidosa (26) was grown under bacteria-free conditions. At the initiation of anaerobic decomposition the culture (TCOD = 1200 mg/l) was divided into two aliquots: one was maintained bacteria-free and the other was seeded with 10 ml per liter of a previously decomposing culture (Culture 9) containing bacteria.

The results from the stabilization of TCOD in these cultures during decomposition is presented in Figure 6A. As expected, no significant stablization of TCOD occurred in the bacteria-free culture, while a relatively rapid rate of stabilization was accomplished by the methane bacteria in the other culture. The small amount of stabilization in the bacteria-free culture can be explained as resulting from the possible production of H_2 by the Chlorella, a process that this alga is known to be able to carry on to a limited degree under the conditions of the experiment.

The reduction in PCOD in the two cultures is illustrated in Figure 6B and indicates that a significant degree of conversion of particulate organics to soluble organics was accomplished in the bacteria-free culture. The extent of reduction in the pure cultures was almost as great

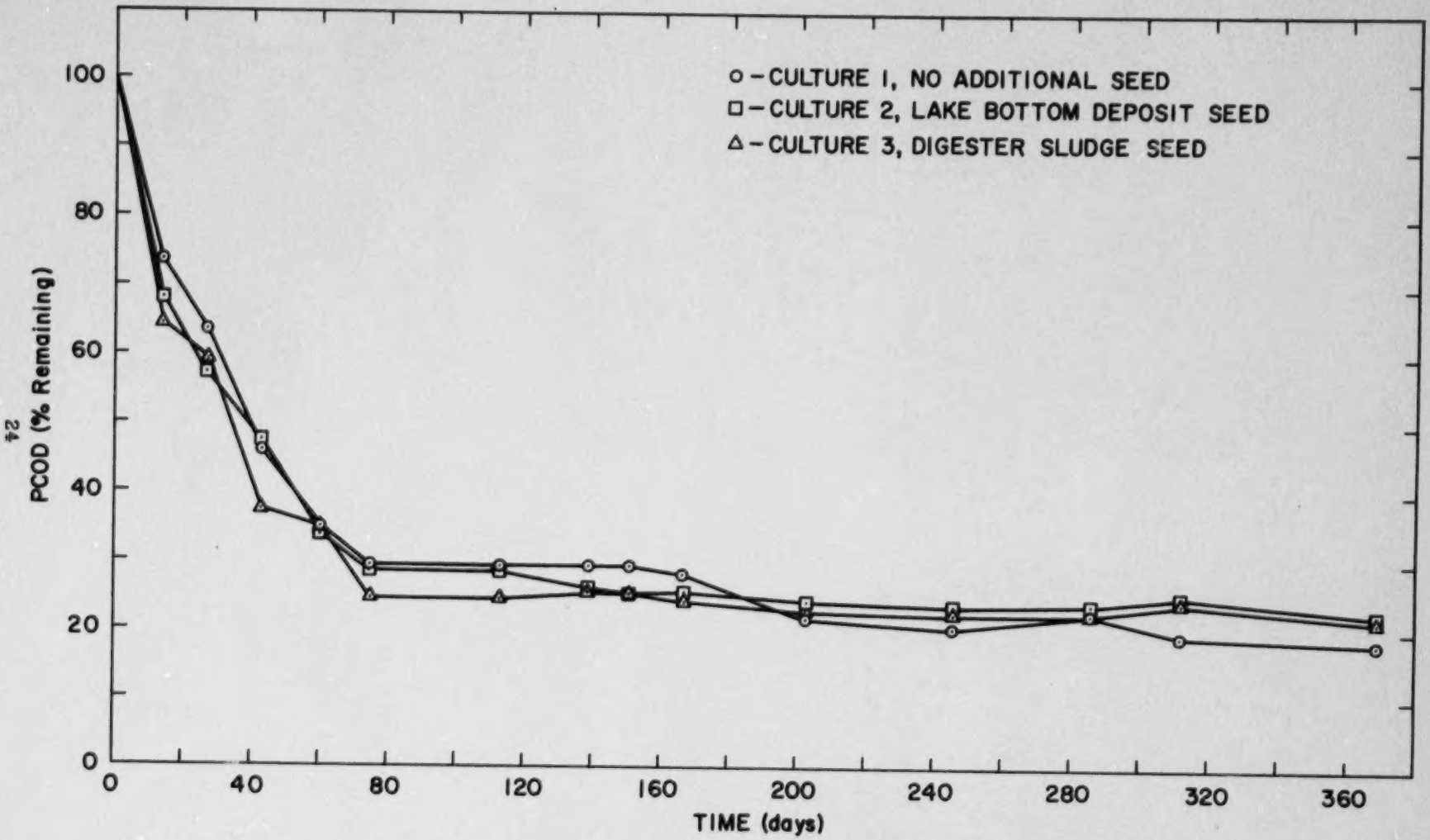

FIGURE 5. Effects of Bacterial Seeding on PCOD Reduction in Decomposing Cultures of Pond Algae.

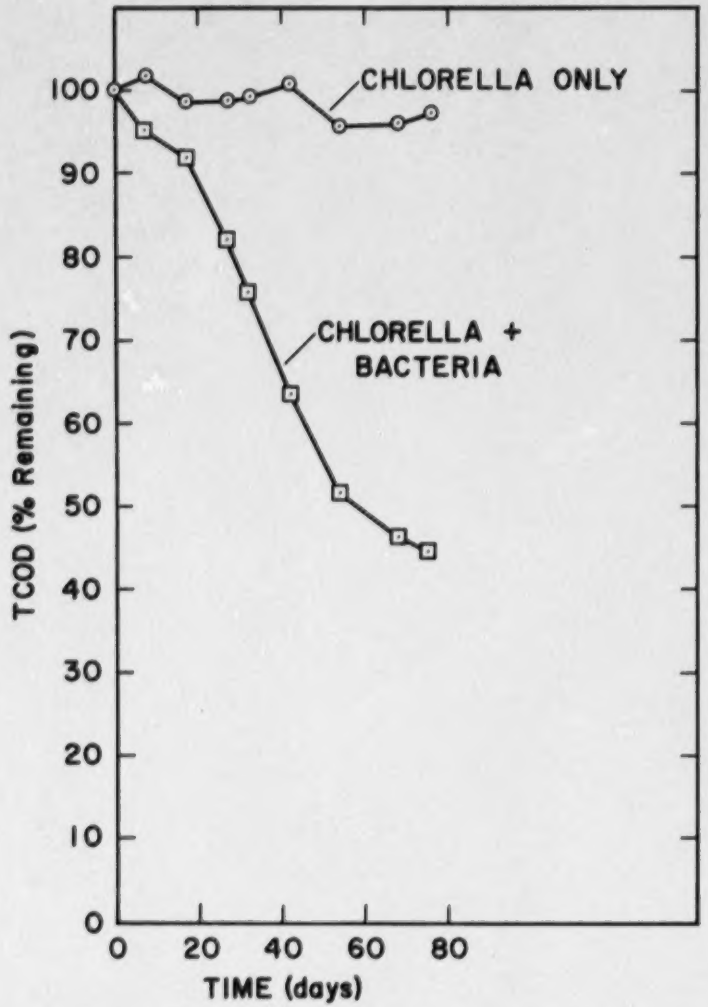

FIGURE 6A. Effect of Bacteria on the Stabilization of TCOD in Decomposing Chlorella Culture.

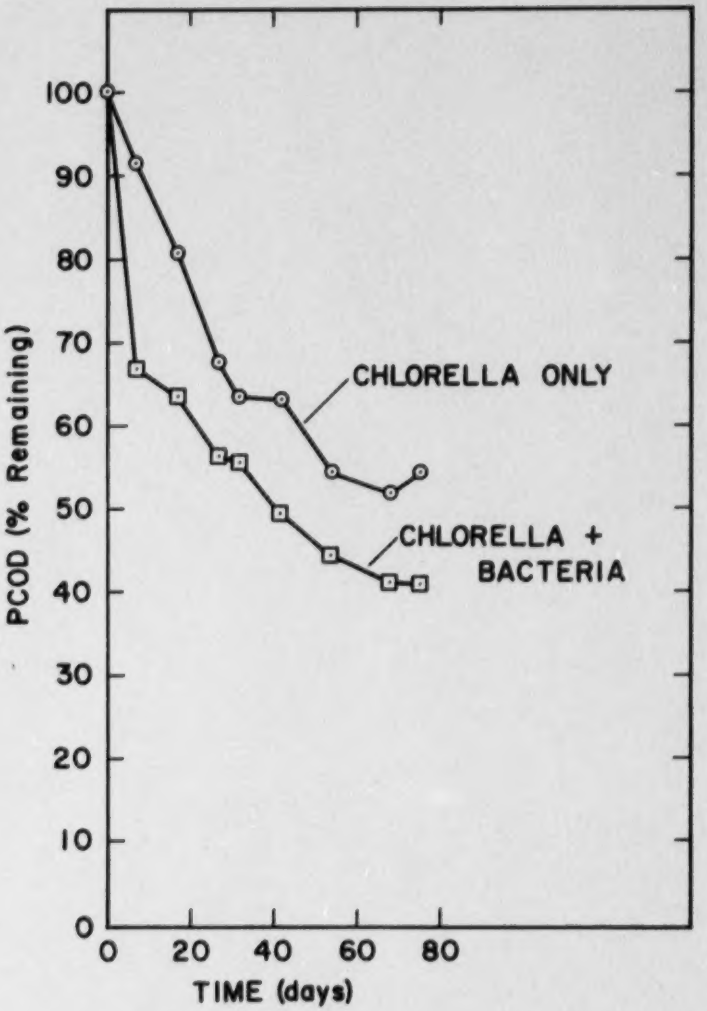

FIGURE 6B. Effect of Bacteria on Reduction of PCOD in Decomposing Chlorella Culture.

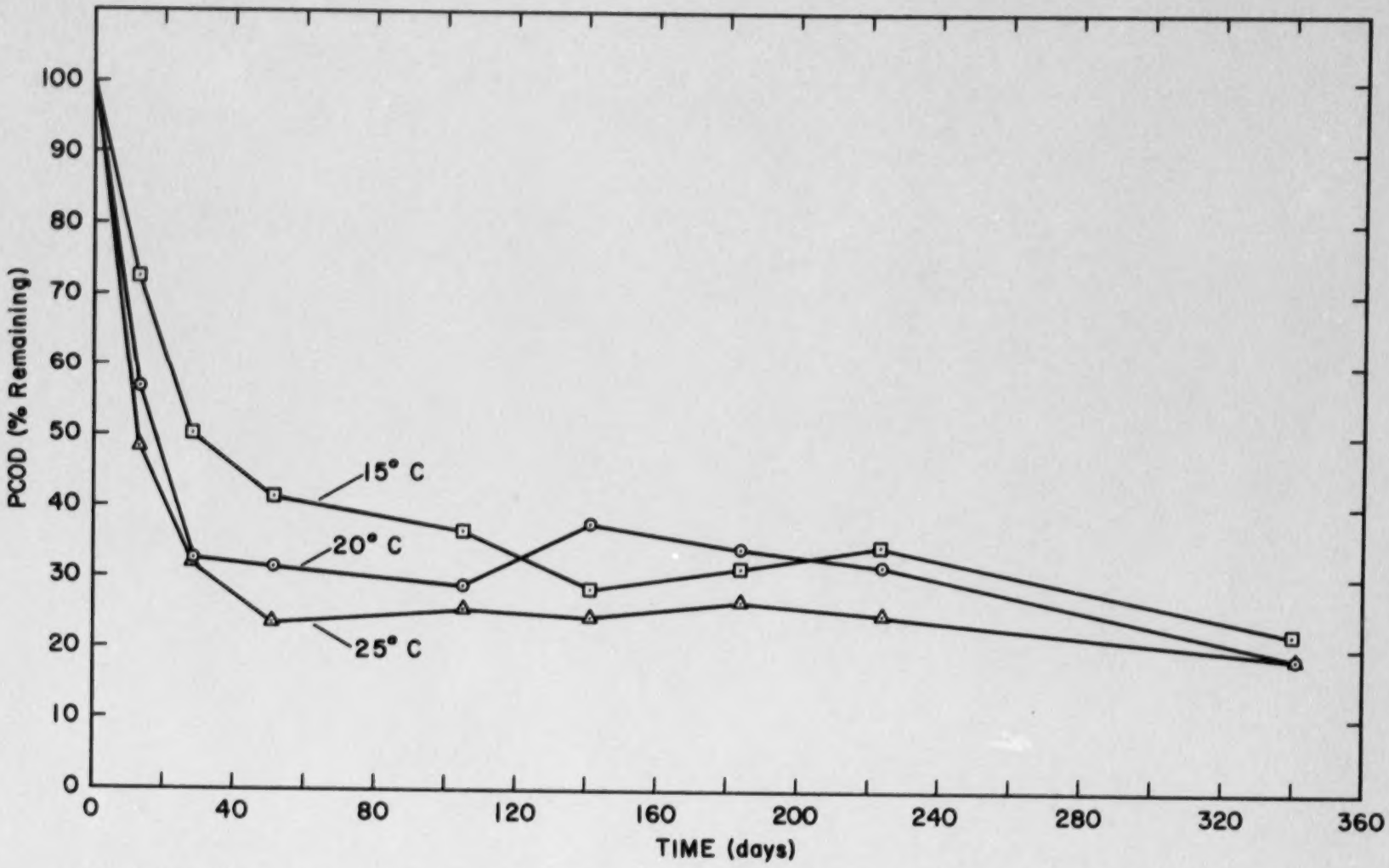

FIGURE 7. Effects of Temperature on PCOD Reduction in Decomposing Cultures of Pond Algae.

as in the seeded cultures indicating the algae themselves bring about a significant portion of the conversion to soluble organics. However, the results of volatile acids analyses showed that no volatile acids were produced in the bacteria-free culture, while most of the soluble organics were represented as volatile acids in the seeded cultures. This indicates bacteria are necessary to convert the soluble organics released to volatile acids.

2. Temperature. A sample of algae was collected from a pond, suspended in a sythetic medium, and divided into three aliquots. Each aliquot was seeded with 1.7 ml per liter of a sample taken from a previously decomposing culture (Culture 4). The cultures were incubated under dark anaerobic conditions at three different temperatures: 20° C (Culture 9), 15° C (Culture 10), and 25° C (Culture 11).

A plot of the reduction of PCOD during decomposition is presented in Figure 7. Although the rate of decrease in PCOD was higher during the early stages of decomposition at the higher temperatures, the extent of decomposition after about 60 days was not significantly different in the various cultures. Thus temperature was found to affect the rate, but not the extent of decomposition.

A plot showing the stabilization of TCOD with time in these cultures is presented in Figure 8. A significant decrease in the degree of stabilization with decrease in temperature was observed over about the first 200 days of decomposition, but the long-term extent of stabilization again was not significantly different for the three temperatures studied.

The results of volatile acids analyses shown in Figure 9 indicate that the decrease in rate of stabilization with temperature could be attributed mainly to decrease in the rates of utilization of volatile acids by the methane bacteria. While the rate of utilization of acetic acid was affected by temperature, the utilization of propionic acid was affected

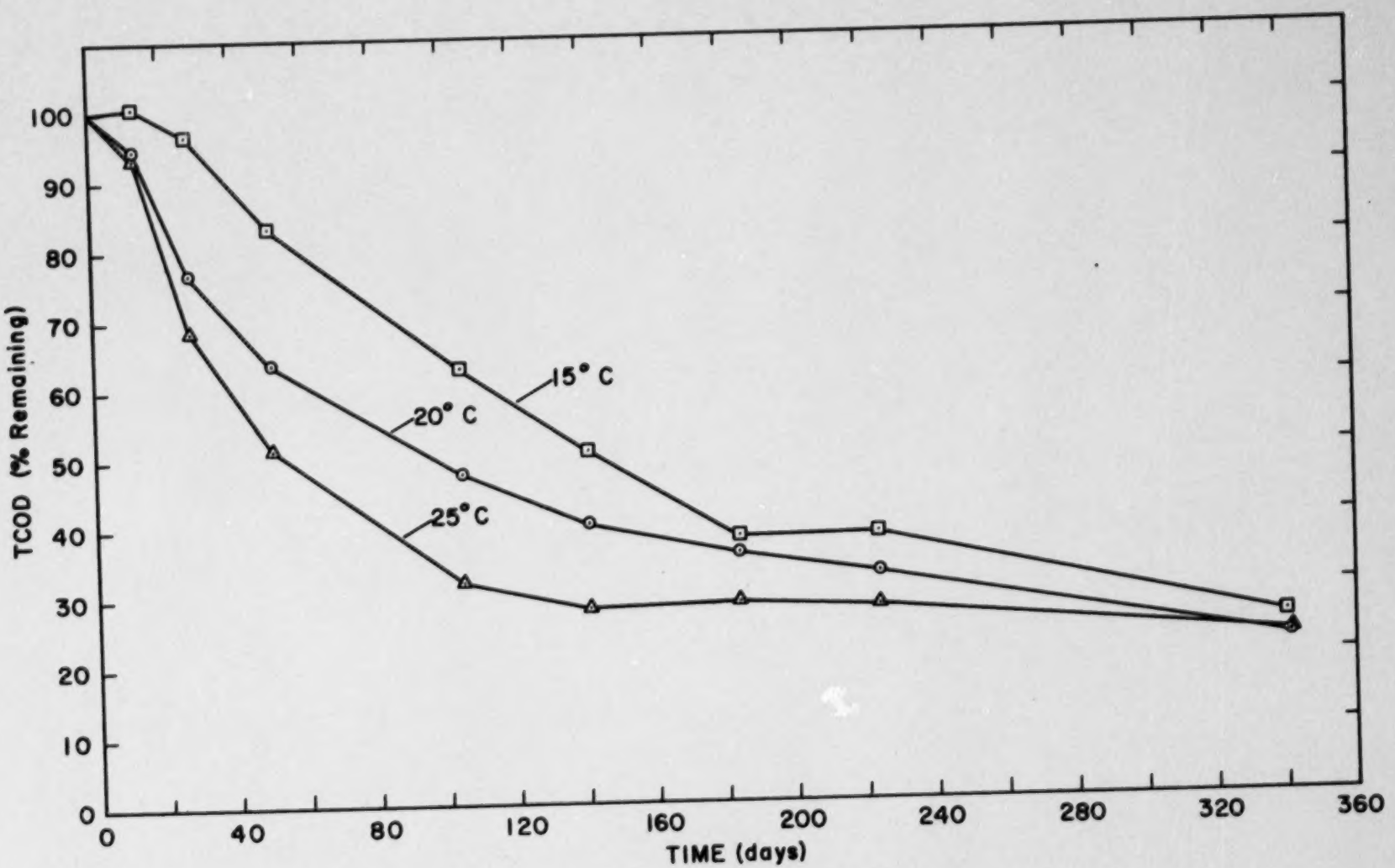

FIGURE 8. Effects of Temperature on TCOD Stabilization in Decomposing Cultures of Pond Algae.

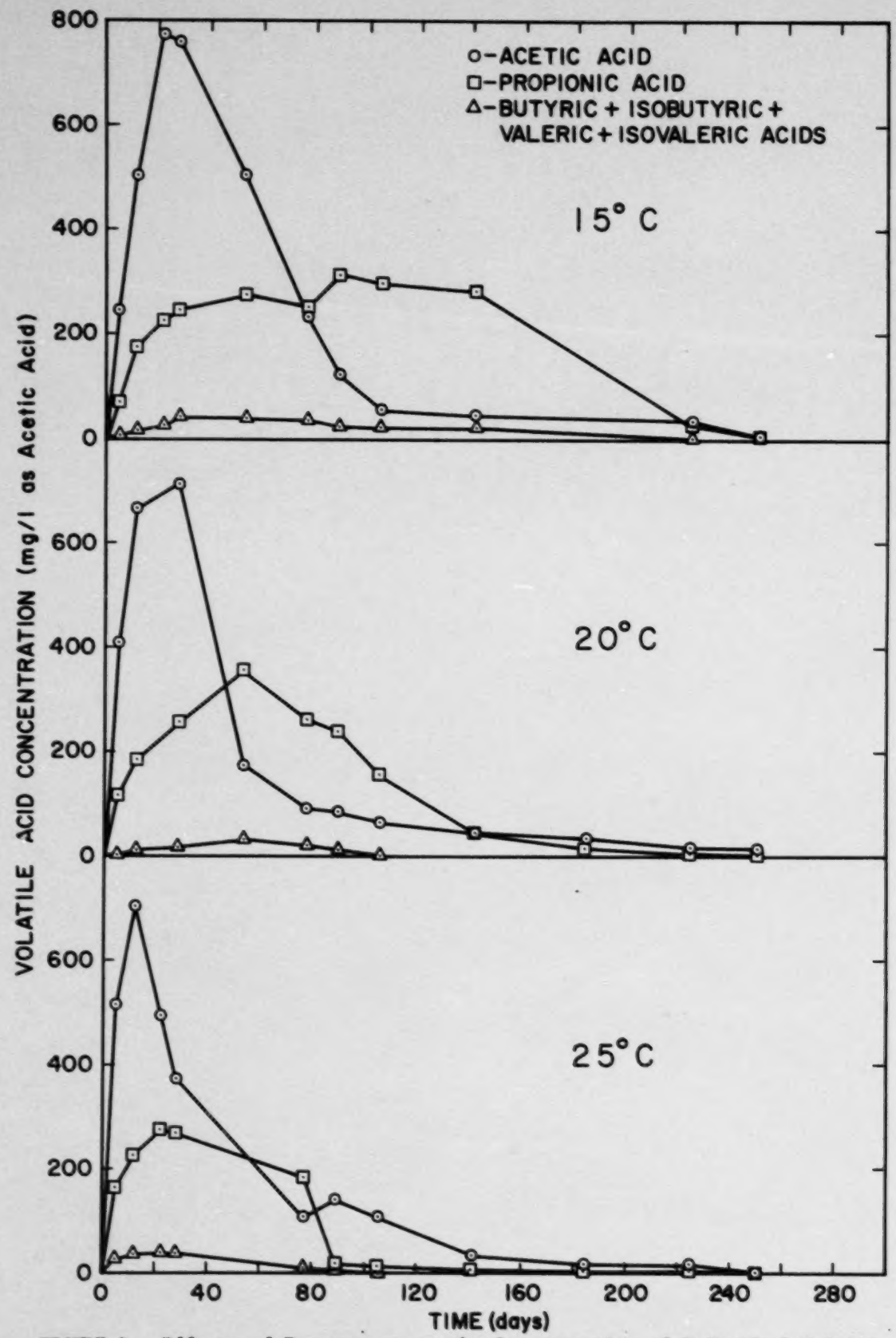

FIGURE 9. Effects of Temperature on the Concentration of Individual Volatile Acids Present in Decomposing Cultures of Pond Algae.

to a relatively greater degree. This is consistent with the results found by O'Rourke (6), for the methane fermentation of complex waste sludges.

3. pH. The initial rate of production of volatile acids was normally greater than their initial rate of utilization by the methane bacteria, resulting in a build up in the concentration of the acids. This, in turn, can result in a decrease in pH in the medium, if the buffering capacity is not sufficient, to a level which is toxic to the methane bacteria so that little or no methane fermentation and hence stabilization will occur.

The phenomenon was observed in two cultures in which the initial net rates of production of volatile acids were greater than anticipated as shown in Figure 10. Culture 112 which was used in this study was an unialgal culture of Lyngbya sp. and was seeded at the beginning of decomposition with 10 ml per liter of municipal wastewater. Less than 10 percent stabilization of TCOD was accomplished in this culture, after over 200 days of decomposition. However, about 70 percent reduction in PCOD did occur. All of the soluble organics formed as the result of the reduction in PCOD were present in the form of volatile acids, and almost one-half of the total acids produced were produced during the period when the pH was 4.6-4.9, indicating that the volatile acids production was not seriously inhibited by pH levels this low.

Another study was conducted to further investigate the effects of pH on decomposition. A heterogeneous culture of algae was grown in a synthetic medium containing a bicarbonate alkalinity of 200 mg/l (as $CaCO_3$). At the beginning of the decomposition study, the culture was divided into four aliquots and the alkalinity was adjusted to the following values using $NaHCO_3$ and HCl: Culture 170 - 50 mg/l, Culture 171 - 200mg/l, Culture 172 - 500 mg/l, and Culture 173 - 1500 mg/l. This would permit a different pH to result in each culture during fermentation. NaCl was added to make the Na concentration equal in all cultures to correct for any possible effects due to cation inhibition.

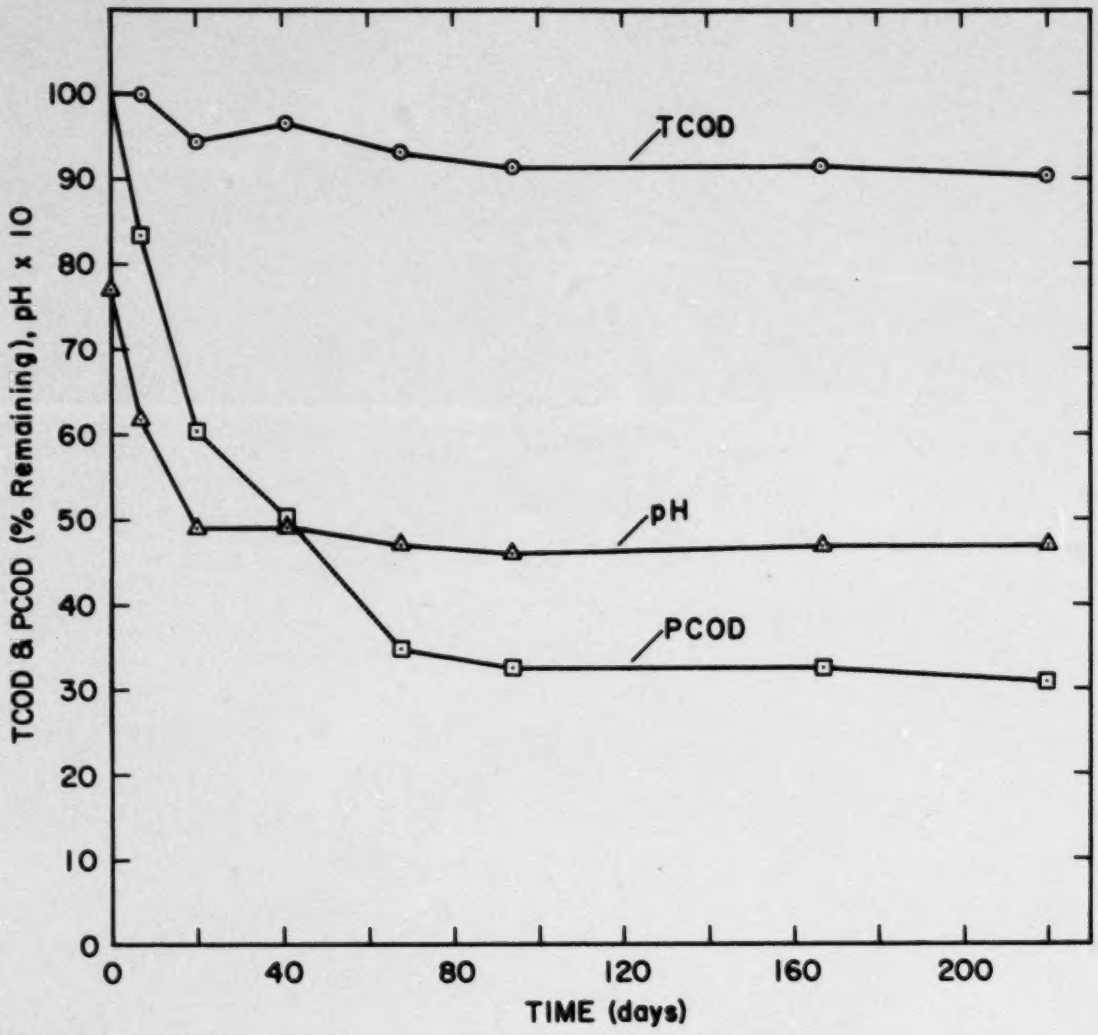

FIGURE 10. Effects of Low pH on Stabilization of TCOD and Reduction of PCOD in a Decomposing Culture of Lyngbya sp. (622).

The results indicated in Figure 11 show that no significant TCOD stabilization (methane fermentation) was accomplished in the two cultures in which the pH levels decreased below about 5, but was obtained in the two cultures in which the pH remained above 6.3. The reduction of PCOD was not significantly different in the three cultures in which the pH remained above 4.4, but was inhibited in the culture with pH of 3.2. The results of volatile acids analyses indicated that this lowest pH had an inhibitory effect on the acid-forming bacteria as well as the methane formers. Essentially all of the soluble organics produced in the three higher pH cultures were converted to volatile acids, while only a small portion was converted in the culture with pH of 3.2. Although negligible methane fermentation occurred in Culture 171 (minimum pH, 4.6), the volatile acids production was not significantly affected at this pH. This is in agreement with the results presented in Figure 10 for Culture 112.

4. Algal Species. A study was conducted to determine if there were significant differences in the decomposition characteristics among various common species of algae under similar conditions. The algae for these studies were grown as unialgal cultures and were seeded with 10 ml per liter of settled municipal wastewater at the beginning of the decomposition studies.

Results for three species of green algae are presented in Figure 12, and for three species of blue-green algae, in Figure 13. Reduction in PCOD only is presented here because it is the parameter which is more directly related to the characteristics of the algae undergoing decomposition.

The results indicate that there are some differences in both the rate and the extent of reduction of PCOD among the various species of algae. However, the results of other studies indicate that, in general, effects due to variations in cell composition and environmental factors are far more significant than those related to the identity of the algal species per se.

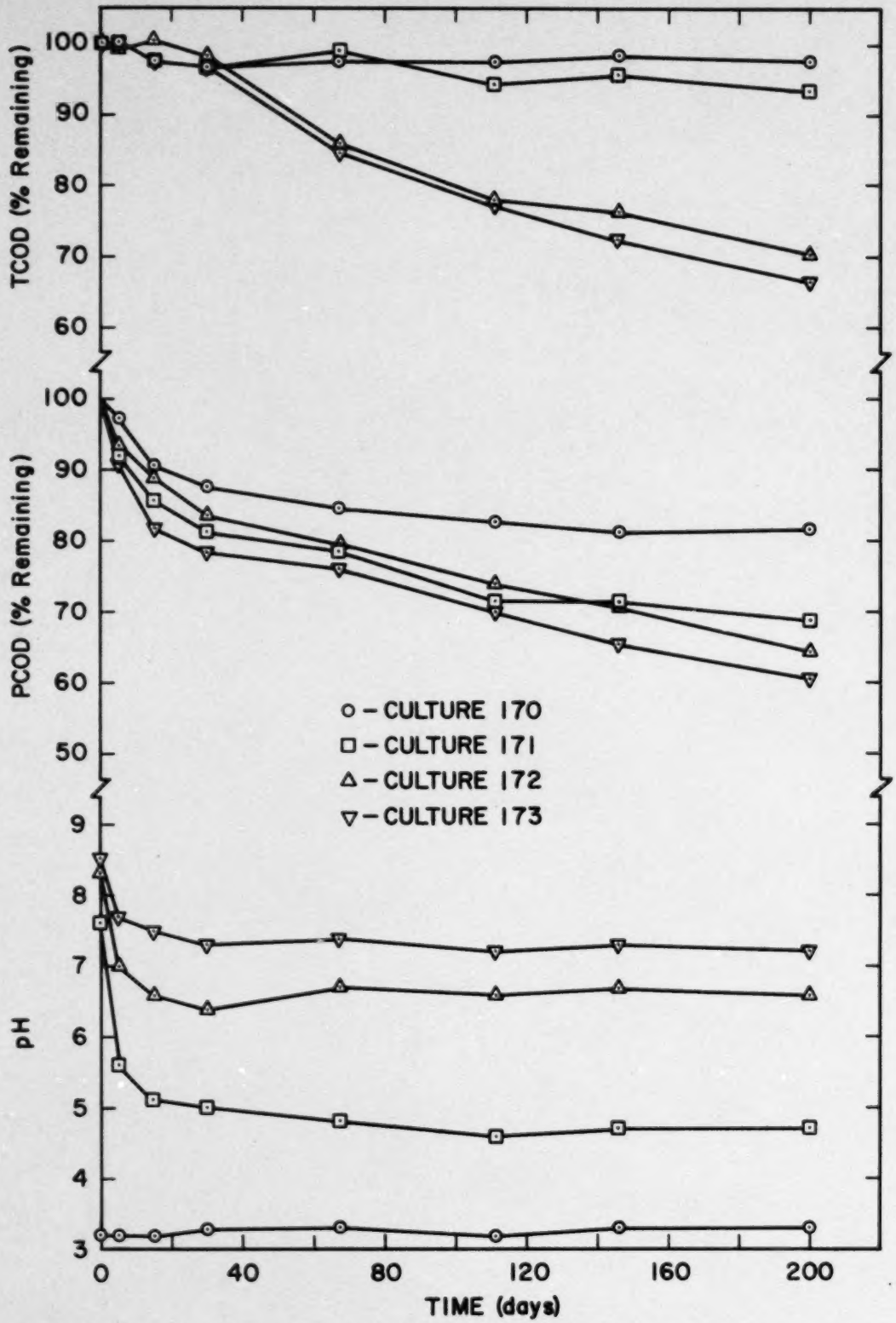

FIGURE 11. Effects of Variations in pH on TCOD Stabilization and PCOD Reduction in Decomposing Heteregeneous Cultures of Algae.

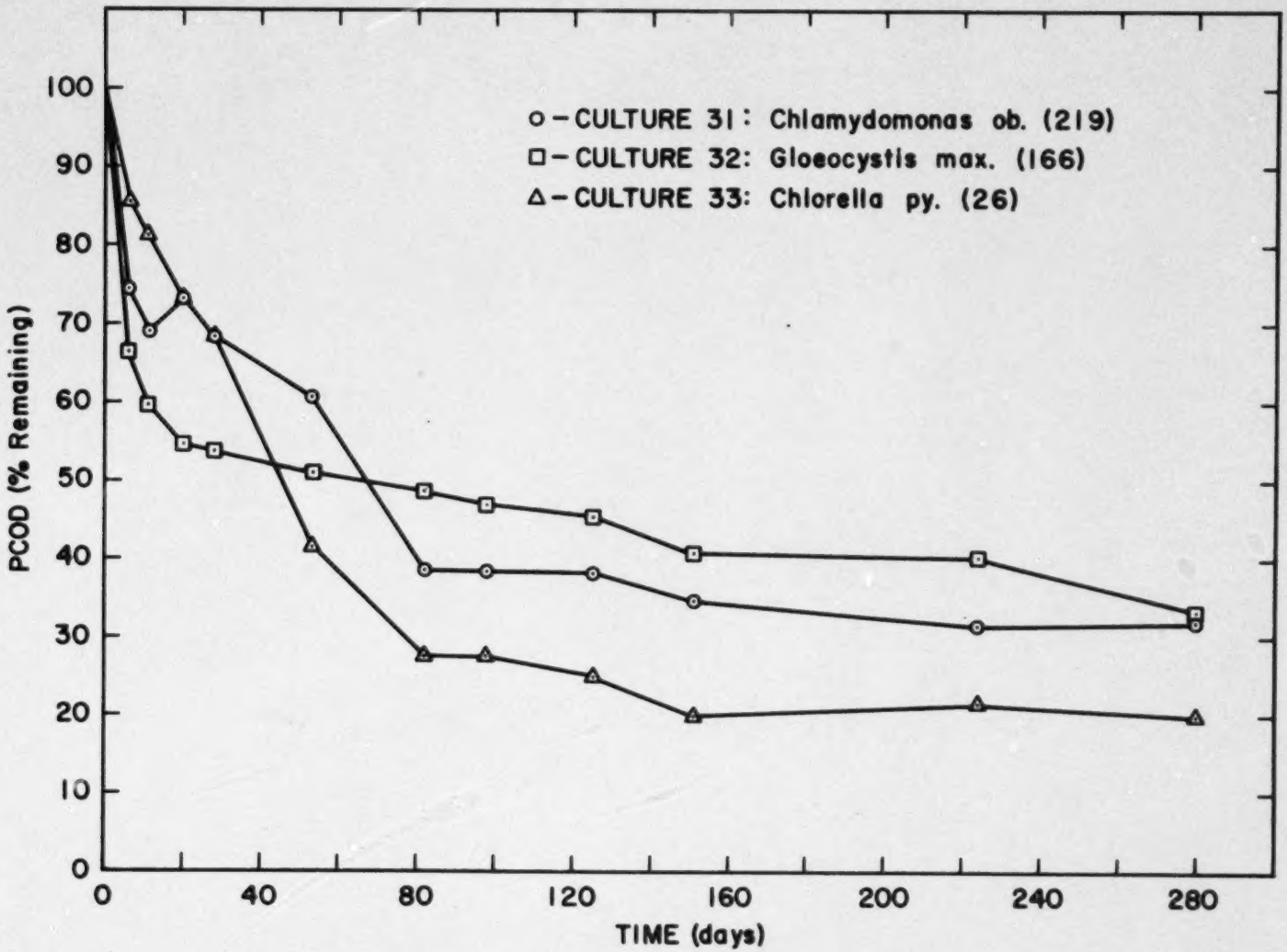

FIGURE 12. Variations in Reduction of PCOD in Three Decomposing Cultures of Green Algae.

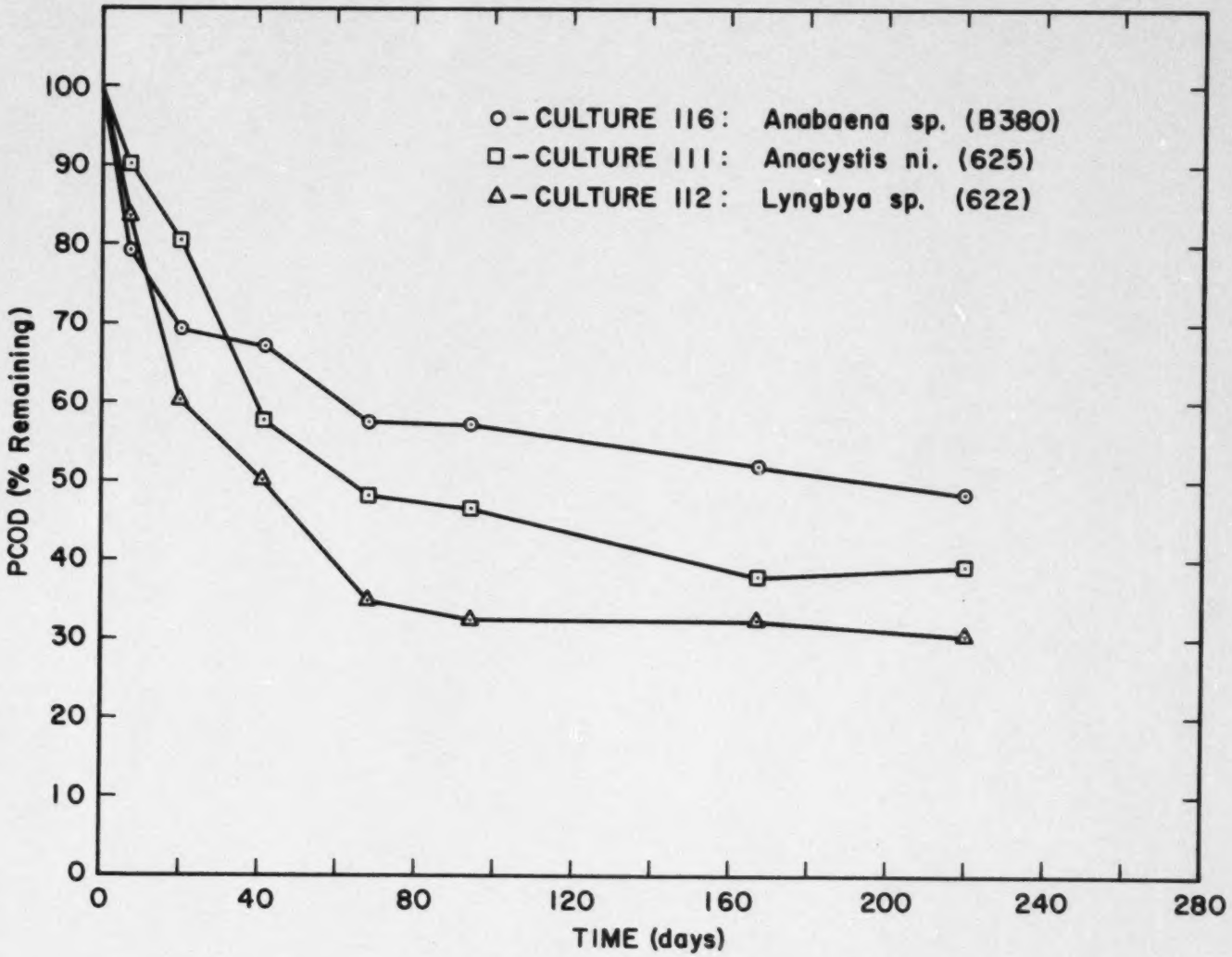

FIGURE 13. Variations in Reduction of PCOD in Three Decomposing Cultures of Blue-Green Algae.

5. Algal Cell Composition. Some of the most significant effects on decomposition resulted from variations in cell composition. The most striking differences observed were associated with variations in the lipid content. Other investigators have shown that the lipid content can vary over a wide range, especially for several species of green algae. Spoehr and Milner (1) found lipid contents increasing from 4.5 percent to 86.3 percent on an ash-free dry weight basis for Chlorella py, primarily as a response to the exhaustion of the nitrogen supply in the medium. The cell composition normally remained relatively constant as long as nitrogen was available in the medium; once this nitrogen supply was exhausted, lipids accumulated, while the absolute quantities of proteins and carbohydrates remained relatively constant, resulting in an increase in the percentage of lipids. Lipid accumulation is not characteristic of all algae: some algae accumulate carbohydrates to varying degrees and several important blue-green algae are capable of the fixation of N_2 so that their cell composition can remain relatively constant even when the combined nitrogen supply in the medium is exhausted.

The importance of lipid content in relation to anaerobic decomposition lies in the fact that; (1) lipids are insoluble and are measured as PCOD even if released by the cell, and (2) while the acid-producing bacteria can convert proteins and carbohydrates to soluble volatile acids, the methane bacteria are solely responsible for the degradation of lipids. Thus, no significant reduction in the lipid PCOD can be accomplished without methane fermentation and hence TCOD stabilization.

The stabilizations of TCOD and reduction of PCOD during decomposition of two cultures of Chlorella pyrenoidosa, one with a low lipid content and one with a high lipid content, are shown in Figure 14. The cell compositions for these cultures at the beginning of decomposition are presented in Table 5.

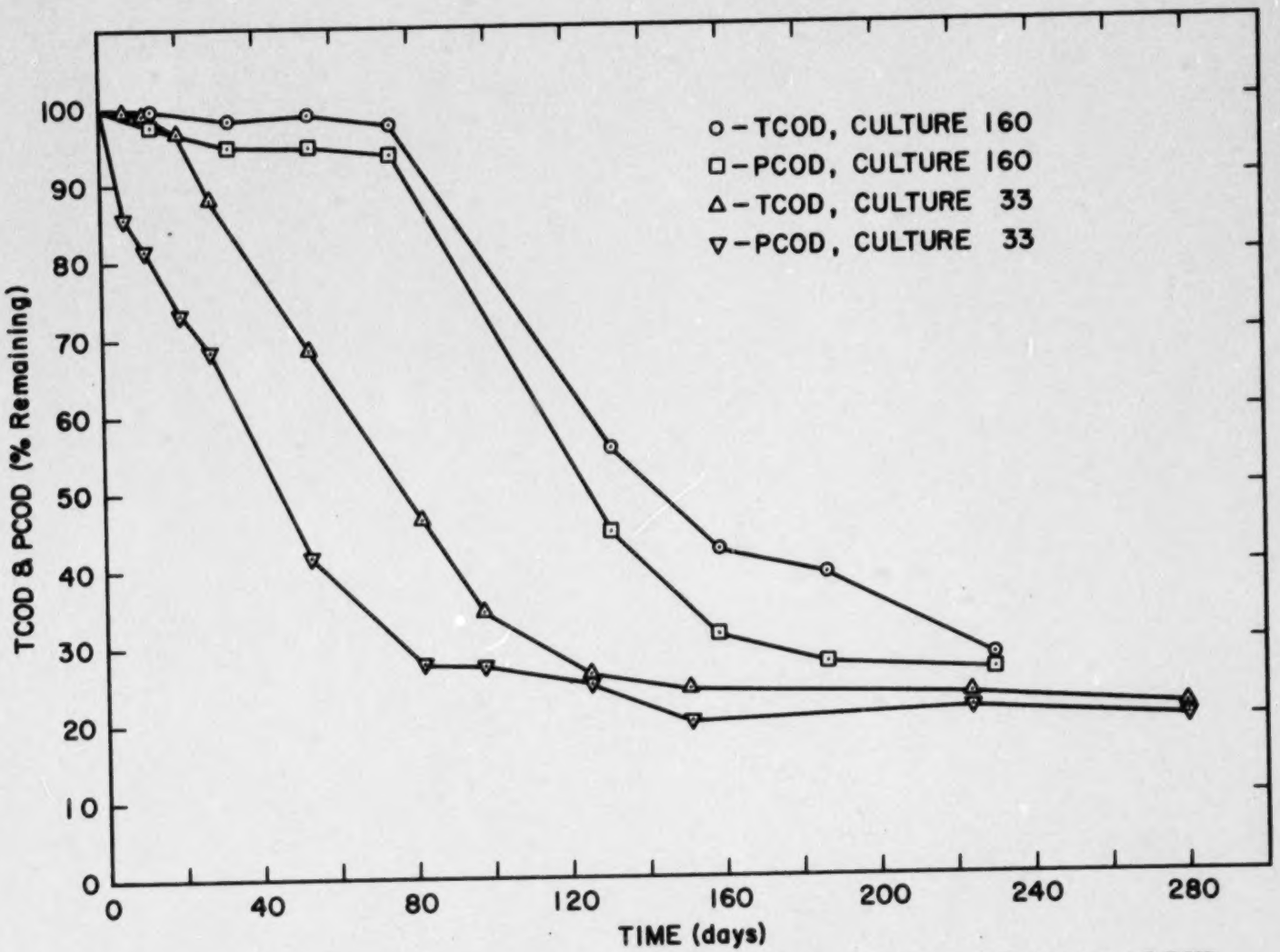

FIGURE 14. Effects of Lipid Content on Stabilization of TCOD and Reduction of PCOD in Two Decomposing Cultures of Chlorella py. (26).

TABLE 5

COMPOSITION OF CHLORELLA PYRENOIDOSA AT BEGINNING OF ANAEROBIC DECOMPOSITION

Culture	(% Ash-free Dry Weight) Protein	Lipid	Carbohydrate
33	51.4	10.7	37.9
160	11.2	62.5	26.3

The results are not exactly comparable as they reflect the combined effects of both bacterial seeding and algal cell composition. Culture 33 was seeded with 10 ml per liter of settled municipal wastewater and no bacterial seed was added to Culture 160 except that present in the water used to make up the growth medium. However, as previously shown, the effect of this difference in seeding does not usually affect PCOD reduction but does result in a longer initial lag in TCOD stabilization (methane fermentation).

The required presence of methane bacteria for the reduction of PCOD in Culture 160 (high lipid content) is clearly demonstrated by the relatively small reduction during the first 75 days, and the subsequent reduction in PCOD coincident with stabilization of TCOD (methane fermenation). Conversely, in Culture 33 (low lipid content) about 30 percent reduction in PCOD was accomplished before significant stabilization of TCOD began to occur.

D. Long-Term Degree of Degradation of Algal Organics and Composition of Refractory Material.

Two independetnly determined measurements of the quantity of particulate material, PCOD and VSS, were considered to be the best parameters to use as a measure of the degradability of the algal organics. As previously demonstrated, stabilization of TCOD is dependent upon methane fermentation and thus, in general, is not as closely related to the characteristics of

the algal material as is the reduction in PCOD or VSS. The quantities of PCOD and VSS (expressed as percentages of the initial values) remaining after 200 days of decomposition are presented in Table 6 for 20 cultures. Included in the table are all of the cultures studied that met the following qualifications: (1) decomposition observed for at least 200 days, (2) decomposition temperature, 20° C, (3) TCOD stabilization accomplished by methane fermentation, and (4) pH remained above 5.5.

Two hundred days was chosen as a convenient common point in time for comparison of the various cultures. In general, no significant decrease in PCOD or VSS was found to occur after this period of time.

TABLE 6

QUANTITIES AND CHARACTERISTICS OF REFRACTORY MATERIAL REMAINING IN 20 ALGAL CULTURES AFTER 200 DAYS OF DECOMPOSITION

Culture	PCOD (% Remaining)	VSS	(PN/VSS)x100 (%)	PCOD/VSS (mg/mg)
1	22.3	17.4	7.18	1.28
2	24.4	24.5	5.12	1.14
3	22.9	19.6	5.49	1.22
4	17.8	16.7	6.59	1.07
9	33.1	25.2	5.56	1.43
31	32.5	29.4	9.90	1.63
32	40.2	37.5	2.94	1.12
33	21.0	16.1	12.10	1.93
110	45.0	46.1	4.52	1.75
111	39.0	42.8	11.41	1.50
115	43.8	32.2	9.24	1.88
116	50.0	47.8	4.44	1.32
144	57.3	56.4	2.21	1.34
160	27.0	28.9	5.30	2.04
162	46.0	47.8	3.70	1.36
163	57.8	57.7	5.53	1.43
164	58.4	58.3	5.07	1.51
166	61.0	59.9	5.20	1.42
172	64.3	57.6	3.70	1.57
173	60.3	54.7	3.87	1.62
Range	18-64	16-60	2.2-12.1	1.1-2.0
Mean	41.2	38.6	5.97	1.48

For example, decomposition was observed in Culture 3 for 613 days, the longest time of any culture studied with the results shown in Table 7.

TABLE 7

FRACTION OF PCOD AND VSS REMAINING IN CULTURE 3
AFTER EXTENDED PERIODS OF DECOMPOSITION

Time (days)	PCOD (% Remaining)	VSS (% Remaining)
200	22.9	19.6
613	20.4	17.9

Included in Table 6 are 5 cultures of algae collected from ponds, 8 unialgal cultures, 4 heterogeneous cultures grown in synthetic media, and 3 cultures grown in natural waters. Therefore, the mean value for both PCOD and VSS of about 40 percent is considered to be relatively good "average" expected value for the quantity of refractory material remaining under the specified conditions. A much better prediction can be made in a particular situation with a knowledge of the prevalent environmental and biological factors previously discussed.

Also presented in Table 6 are the nitrogen contents (expressed as percent of ash-free dry weight) and PCOD:VSS ratios of the refractory material after 200 days of decomposition. Along with certain assumptions, these two factors can be utilized to calculate the composition of the algal organics with respect to protein, lipid, and carbohydrate content. The assumptions are that the protein, lipid, and carbohydrate fractions each have typical chemical compositions, and that the sum of the protein, lipid, and carbohydrate fractions equals 100 percent of the ash-free dry weight (measured as VSS). These assumptions permit the composition to be calculated as follows (all percentages on an ash-free dry weight basis):

$$\% \text{ Protein} = 6.25\ (\% \text{ N})$$

$$\% \text{ Lipid} = [100R - 107 - 0.43\ (\% \text{ Protein})]/1.79$$

$$\% \text{ Carbohydrate} = 100 - \% \text{ Protein} - \% \text{ Lipid}$$

where,

$\% N$ = Measured % Nitrogen, and

R = Measured PCOD:VSS ratio.

It was necessary to use a formulation of this type because the lipid contents were not routinely measured directly in most of the cultures. Close agreement was obtained between the lipid contents calculated in this manner and those determined by direct measurements for the cultures in which lipids were measured directly. These cultures had a wide range of lipid contents and moderate protein and carbohydrate contents. However, in cultures with very high carbohydrate contents (indicated by a low PCOD:VSS ratio) the applicability of the above formulation is limited. Nevertheless, a composition calculated using the above formulation is considered to give a relatively good estimate of the average composition of the refractory material.

Using the mean values indicated in Table 6, the following "average" composition was calculated: protein - 37.3 percent, lipid - 14.0 percent, and carbohydrate - 48.7 percent. Utilizing an identical procedure, the following initial "average" composition for this same group of cultures was calculated: protein - 26.6 percent, lipid - 11.0 percent, and carbohydrate - 62.4 percent. These results suggest that after about 60 percent degradation, the composition of refractory material is similar to that of the initial material, or at least that there is no striking difference. The increase in protein content during decomposition can be explained, at least in part, by an increase in the quantity of refractory bacterial solids which occurs during decomposition. Bacteria are known to have a considerably higher protein content (about 70 percent) than the average value for the initial algal material (26.6 percent).

DISCUSSION

The study to date on the decomposition of algae under anaerobic onditions has shown several important factors which can affect water quality. The rate and extent of conversion of particulate algal material to soluble organics was about the same as observed under aerobic conditions. The major exception was in the decomposition of algae with a high lipid content. In this case, decomposition and stabilization proceeded together and required the presence of methane producing or sulfate reducing bacteria.

The soluble organic material released by anaerobic decomposition may be stabilized by any of several different processes. It may be carried up by diffusion or turbulance to an aerobic zone and there be oxidized by normal facultative organisms. This would adversely affect the dissolved oxygen resources. In the absence of dissolved oxygen, other electron acceptors such as nitrates, sulfates, or carbon dioxide may be used by microorganisms while stabilizing the soluble organic matter. If nitrates are used, the nitrate nitrogen will be reduced to nitrogen gas and the nutrient supply of the water will be reduced. If sulfates are used, the sulfur will be converted to hydrogen sulfide gas which will increase water odor. The hydrogen sulfide may also diffuse, be carried up in bubbles, or may be transported by other menas to aerobic waters where it can be oxidized autotrophically, again resulting in a reduction in dissolved oxygen. Finally, if carbon dioxide is used as an electron acceptor, methane gas will be produced. This gas is quite insoluble and a good portion may escape from the water without affecting the dissolved oxygen resources. In certain situations, however, it too can be oxidized by certain bacteria and this would adversely affect the dissolved oxygen content.

In any event, it is desirable to know the rate at which the soluble organics would be produced from decomposing algae in order to assess the possible significance of any of the above processes on water quality. This aspect has received and will continue to receive the major

emphasis in this research investigation. Methane fermentation and sulfate reduction can be important and are being studied to some extent. However, the rates of these reactions are fairly well known and very dependent upon the quantity of fermenting organisms present.

Studies on the effect of a high sulfate concentration on the anaerobic decomposition of algae is presently under investigation. The results to date indicate that methane fermentation will not be significant under such conditions as the hydrogen sulfide produced is sufficiently high in concentration so that it is toxic to the methane bacteria. It has also been noted that in the presence of sulfate reduction, acetic acid was the only soluble compound present in significant concentration, and persisted for some time before sulfate reducing bacteria began its metabolism.

Parallel and continuing studies are also concerned with the regeneration of nitrogen and phosphorus under anaerobic conditions. The results to date indicate a similar trend to that found in the aerobic decomposition studies. The extent of regeneration is related to the concentration of nutrients present in the algae at the time regeneration begins. Nutrient rich cells permit regeneration of a large fraction of the nutrients, while the reverse is the case with nutrient poor cells. In all cases however, a significant fraction of the nitrogen and phosphorus remains in the refractory fraction of the algae after decomposition is essentially completed.

Research in these latter areas is continuing and should be completed sometime during the last year of proposed research. These data plus data already available on the rates of decomposition under a variety of anaerobic conditions will be analyzed and summarized in a more complete final report. This can all be accomplished during the coming final year of proposed research.

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